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Genetically and Epidemiologically Related "Non-Syncytium-Inducing" Isolates of HIV-1 Display Heterogeneous Growth Patterns in Macrophages

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The objective of this study was to identify phenotypic parameters that could distinguish among seemingly homogeneous non-syncytium-inducing (NSI) viruses and that might provide a surrogate marker for clinical progression in pediatric human immunodeficiency virus type 1 (HIV-1) infection. We undertook a pilot analysis of 15 independent HIV-1 isolates collected prospectively from two mothers and their four children who displayed a spectrum of disease stages ranging from CDC categories A1 to C3. Viruses were evaluated for their ability to replicate in primary cells (including monocyte-derived macrophages [MDM]) and cell lines, for their co-receptor preference and for genetic features of the V3 hypervariable domain of *env*. Virtually all isolates displayed NSI phenotypes that were restricted in their capacity to replicate in cell lines and displayed V3 loops with uniformly low net positive charges. NSI viruses from two symptomatic children and one mother were macrophage-tropic, whereas NSI isolates from two asymptomatic children were unable to replicate in MDM and were designated primary lymphotropic viruses. Only one isolate was syncytium-inducing (SI), replicated in a variety of cell lines and in MDM, used multiple co-receptors, and was dual tropic, rather than a mixture of T-cell tropic and M-tropic viruses, as assessed by genetic analysis. Phenotypic heterogeneity among NSI viruses is revealed in the ability of isolates to replicate in MDM. This characteristic is related to disease stage and provides a potentially new in vitro criterion to distinguish among NSI isolates that is unlinked to other surrogate markers. ***J. Med. Virol. 61:171–180, 2000.***

KEY WORDS: viral phenotype; viral isolates; pediatric HIV infection; monocyte-derived macrophages; non-syncytium-inducing; dual tropism

BACKGROUND

The pathogenic potential of strains of human immunodeficiency virus type 1 (HIV-1) and the relationship between biologic behaviors of the virus and clinical state have been characterized in five different ways: (1) by distinguishing between "slow/low" and "rapid/high" growth in peripheral blood mononuclear cells (PBMC) [DeRossi et al., 1991; Conner et al., 1993; DeRossi et al., 1993]; (2) by assessing the capacity of isolates to form syncytia in MT-2 cells [Koot et al., 1992; Jurriaans et al., 1994]; (3) by observing the use of chemokine co-receptors (principally CXCR4 and CCR5) [Doms and Peipert, 1997; Littman, 1998]; (4) by measuring the charge on the V3 loop of gp120 [Shioda et al., 1992; Sabri et al., 1996; Scarlatti et al., 1993a,b]; and (5) by assessing replication of isolates in monocyte-derived macrophages (MDM) [Levy et al., 1985; Ho et al., 1986]. Some of these biologic behaviors have traditionally been regarded as synonymous, e.g., that syncytium-inducing (SI) strains are T-lymphocytotropic, while non-syncytium-inducing (NSI) strains are macrophage-tropic; or that macrophage-tropic strains use

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CCR5 exclusively for cell entry, while T-cell tropic strains employ CXCR4 [Tersmette et al., 1988, 1989; Alkhatib et al., 1996]. Recent evidence suggests that there are frequent exceptions to these traditional biologic equivalencies [Simmons et al., 1996, 1998; Zhang et al., 1996; Dittmar et al., 1997]. The dichotomous division of strains of HIV into SI and NSI phenotype has prognostic significance in adult patients but falsely implies the occurrence of abrupt "switch" mechanisms. In addition, the strict division of viral isolates into SI and NSI has little applicability to the pediatric form of HIV/acquired immunodeficiency syndrome (AIDS). In children younger than 2 years of age, there is no reproducible correlation between carriage of SI virus strains, p24 antigenemia, $CD4^+$ T-cell count and clinical state [Borkowsky et al., 1994]. Some children remain clinically stable for more than 2 years while carrying SI isolates. By contrast, most younger children may progress to AIDS while carrying NSI strains of HIV [Spencer et al., 1994; Fitzgibbon et al., 1998]. These findings point to the likely presence of biologic diversity among NSI isolates collected from children and indicate that some NSI isolates play a role in disease progression. Beyond explorations of the relationship between the syncytium-inducing capacity of HIV isolates and clinical progression of pediatric HIC disease, only limited assessments have been made of the relationship between other biologic behaviors and their association with disease pathogenesis. Of published studies, most have been cross-sectional analyses of unrelated viruses [Borkowsky et al., 1994; Spencer et al., 1994; Fitzgibbon et al., 1998].

We undertook the current studies to learn more about the phenotypic and genetic diversity that exists among NSI isolates derived from children and their mothers. In an effort to identify distinguishing biologic characteristics among the NSI viruses that could be used as markers of pathogenic potential, we focused our analysis on multiple viruses, virtually all NSI, that were isolated prospectively over periods of 2–3 years from siblings and their mothers in two families. This afforded us the opportunity to assess the subtleties of virus phenotype among genetically and epidemiologically related NSI viruses and to examine associations between various biologic behaviors of NSI viruses and disease state. We studied four biologic characteristics of each isolate: (1) the capacity to replicate in primary cells (PBMC and MDM); (2) the ability to replicate in continuous cell lines of varying origin; (3) the ability to induce syncytium formation in PBMC and in continuous cell lines; (4) and the capacity to replicate in HOS cells co-transfected with the CD4 receptor and any of four chemokine receptors: CXCR4, CCR5, CCR2B, and CCR3. We also studied the genetic relatedness among strains, assessed genetic variation following passage through primary cells and examined the concordance between V3 loop amino acid sequence and charge and virus phenotype.

MATERIALS AND METHODS

Patients and Collection of Samples

The patients selected for study were members of two families, each comprising a mother and two children. The fathers were unavailable for study. Characteristics of the six subjects are displayed in Table I. In family 1, the siblings were born 9 months apart; in family 2, they were born 13 months apart. In family 1, all three patients had blood collected for virus isolation in July 1993; two subsequent samples were collected from the younger sib, RM, and one prior and one subsequent isolate were obtained from the older sib, DM. The mother died before a second sample could be collected. All three initial isolates of HIV-1 from patients in family 2 were obtained from blood samples collected on the same day in January 1993; subsequent isolates (one from the mother and two from each child) were collected at intervals ranging from 6 months to 29 months. Altogether, 15 virus isolates were obtained from the six patients.

All studies described were approved by the Human Investigation Committee of Yale University School of Medicine and Yale-New Haven Hospital.

Virus Isolates and Virus Stocks

HIV-1 was isolated from PBMC of each patient by co-cultivation with PBMC from a seronegative donor, using methods that were previously described [Ikeda et al., 1993]. Production of HIV-1 was detected by release of p24 antigen into culture supernatant fluids within 3–7 days (HIVAG Enzyme Immunoassay, Abbott Laboratories, North Chicago, IL). Thereafter, $5–20 \times 10^6$ PBMC from the same seronegative donors used in the primary isolations were added to each culture every 7–10 days in volumes of 10–20 ml in order to propagate the primary virus isolates. Virus stocks were prepared from 50–100 ml of culture supernatant fluids after centrifugation to remove cells and cell debris and filtration through a 0.45-mM Millipore filter; 1-ml aliquots of the stocks were frozen in liquid nitrogen and several representative vials of each stock were titered, using PBMC from seronegative donors as indicator cells. The stocks ranged in titer from $10^2–10^{4.5}$ ID_{50} per ml.

Growth of Viruses in Monocyte-Derived Macrophages

MDM were isolated from fresh donor leukopheresis packs ($>10^9$ cells). PBMC were separated by Ficoll gradient centrifugation; monocytes were further enriched on a subsequent 46% discontinuous Percoll gradient. The enriched monocyte fraction was allowed to adhere to 24-well polystyrene plates at a concentration of 5×10^6 cells per well for 1 hr at room temperature in macrophage medium (RPMI 1640) (Gibco-BRL, Gaithersburg, MD), 15% HuAB serum (Sigma Chemical Co., St. Louis, MO), 2.5 mM Hepes buffer (Gibco-BRL). After 1 hr, monocytes were washed twice, vigorously, to remove any nonadherent cells. To evaluate the purity of MDM populations after 5 days of differentiation in

macrophage medium, cells were stained for nonspecific esterase (Sigma) and analyzed by flow cytometry using monoclonal antibodies CD14 (BD Immunocytometry Systems, San Jose, CA), CD11c (BD Immunocytometry Systems), 2D7 for CCR5 (Pharmagen, San Diego, CA) and 12G5 for CXCR4 (Pharmagen) [Tuttle et al., 1998]. MDM displayed >95% purity. The macrophages were infected with $10^{2.0}$–10^3 ID_{50} of virus stocks prepared and titered in PBMC. The MN strain of HIV-1, which does not grow in MDM culture, was used as a control to demonstrate depletion of T cells from the MDM, whereas the macrophage-tropic Ada-M and JR-FL strains of HIV-1 were used as positive controls. Virus was allowed to adsorb overnight at which time the inoculum was removed, and the cells were washed to remove residual virus. Production of extracellular virus in MDM cultures was measured up to 32 days after infection by monitoring production of p24 antigen levels in supernatant fluids.

Cell Lines

Five cell lines (X50–7.8, H9, MT-2, SupT1,and U937) were used as targets in experiments designed to measure cell tropism and replication of each of the 15 virus isolates. X50–7.8 cells, developed in the Yale laboratory, are Epstein-Barr virus (EBV)-immortalized B cells that are 98% $CD4^+$; they can support the growth of lytic, abortive, and noncytopathic strains of HIV-1 [Dahl et al., 1987]. H9 cells (originally subcloned from the cell line HUT 78), a derivative of a human cutaneous T-cell lymphoma [Popovic et al., 1984]; SupT1 cells derived from a non-Hodgkin's T-cell lymphoma [Smith et al., 1984]; U937 cells derived from a human histiocytic lymphoma [Ralph et al., 1976]; and MT-2 cells, leukemia cells transformed and productive of HTLV-1 virus, were obtained from the AIDS Research and Reference Reagent Program.

Measuring the Biologic Characteristics of Primary Viral Isolates of HIV-1

To measure virus replication, 2×10^6 target cells were infected with 100 ID_{50} of each HIV-1 isolate. After 24 hr, cells were washed multiple times, and the last wash was determined to be free of p24 antigen before they were re-suspended in growth medium, and plated at a cell density of 1×10^6 cells per ml (2 ml per well) in a 24-well plastic dish in a humidified CO_2 atmosphere. Cultures were fed on day 3, after removal of 0.5 ml supernatant fluid. Culture supernatant fluids were collected after centrifugation on days 8 and 11, diluted 10-fold, and assayed for p24 antigen, using a commercial capture antigen immunoassay (HIVAG-1 Enzyme Immunoassay; Abbott Laboratories, North Chicago, IL). In all instances, established cell line cultures were supplemented with 5×10^5 fresh cells of the same type (e.g., SupT1 cells were added to SupT1 cultures, MT-2 cells were added to MT-2 cultures) on day 11, after the removal of supernatant fluids by centrifugation. The cultures were then left undisturbed for 1 week. Supernatant fluids were collected on days 7 and 10 after supplementation of the cultures with fresh cells and were tested for p24 antigen.

The capacity of virus-exposed cells to form syncytia was assayed by counting multinucleated giant cells (using an inverted microscope) on the tenth day after the cultures were supplemented with fresh cells (i.e., day 21 after initiating the cultures).

Assessing Co-Receptor Usage

Approximately 1×10^6 HOS-p.BABE-puro cells either transfected with CD4 alone or co-transfected with CD4 and either CXCR4, CCR5, CCR3, or CCR2b were grown to confluency in six-well plastic plates [Deng et al., 1996; Landau and Littman, 1997]. Cell layers were exposed to 100–1,000 ID_{50} of the first isolate of each of the six patients in volumes of 1.5 ml. After overnight incubation in a 37°C CO_2 incubator, the cell monolayers were washed 5 times with plain Dulbecco's modified Eagle's medium (DMEM) and subsequently maintained in DMEM containing 10% fetal calf serum (FCS) and 1 μg/ml puromycin. Culture supernatant fluids obtained on days 3, 7, and 10 postinfection were assayed for p24 antigen release, and those fluids containing >20 pg per ml of p24 were scored as positive. In some instances, isolates were also assessed for use of co-receptors by measuring viral replication in Jurkat cells transfected with CCR5 or with CXCR4 [Alkhatib et al., 1997]. HOS-pBABE-puro cells not transfected with the CD4 receptor were used as negative controls in evaluating isolates for utilization of CD4 for cell entry. HIV strains MN and 89.6 were used as controls for measuring replication in cells carrying either CXCR4 or both CCR5 and CXCR4 co-receptor, respectively. Macrophage-tropic HIV strain JR-FL was used as a control to assess replication in CCR5-bearing cells.

Virus Genotypes

Genotyping of viruses was carried out by sequencing V3 hypervariable domains of *env* in DNA isolated from infected cells. Cell types included patient PBMC, cultured PBMC, or MDM, and the transformed cell lines of lymphocytic or monocytic origin described earlier.

HIV-1 envelope hypervariable regions V1–V5 were amplified using forward primer 5′ gccacacatgcctgtgtacccaca3′ (nucleotide positions 6432–6456 in the HXB2 genome; Los Alamos Compendium) and reverse primer 5′ cttctccaattctccctcata3′ (7666–7645) in the first round (1234-base pair [bp] product) and a nested forward primer 5′ ggtagaacagatgcatgaggat3′ (6515–6545) with the same reverse primer in the second round (1151-bp product).

First-round amplifications were carried out in 50-μl reactions containing 250–500 ng genomic DNA (quantified by spectrophotometry); 1.75 mM $MgCl_2$, 50 mM KCl, 20 mM Tris (pH 8.2), 100 μg bovine serum albumin (BSA) per ml, 200 μM each deoxynucleotide triphosphate, 1.25 U *Taq* polymerase, and 100 or 500 nM of each primer in the first or second round, respectively. Amplification reactions, which were carried out in a Perkin-Elmer 4800 Tempcycler, included an initial

TABLE I. Clinical Characteristics of Members of Two Families from Whom Sequential HIV-1 Isolates were Obtained

	No. of isolates	Age between first and last specimen	CDC class[a]	Range of $CD4^+$ T-cell counts per μl at time of virus isolations
Family 1				
MM (mother)	1	32 y	C3	<200
DM (child)	3	17–48 mo	B1	1190–2390
RM (child)	3	16–31 mo	A3	374–851
Family 2				
NC (mother)	2	25–27.5 y	A2	250–310
KC (child)	3	32–53 mo	C3	300–678
LC (child)	3	19–40 mo	A1	1538–1695

[a]CDC classes for all patients remained unchanged during the interval between collection of the first and last specimens. Class A, Mild symptoms/signs (A1, without evidence of immunosuppression; A2, with evidence of moderate suppression; A3, with evidence of severe suppression); class B, moderate signs/symptoms (B1, without evidence of immunosuppression); class C, severe signs/symptoms (C3, with evidence of severe suppression).
(CDC. MMWR 1994; 43:No. RR12, 1–19)

denaturation of 95°C for 10 min, followed by 35 cycles each consisting of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 2 min, and a final elongation at 72°C for 10 min. One-tenth of the first-round product was used as the template in a second round of amplification with identical conditions, except that primer annealing was at 60°C.

DNA products were isolated and cloned into TA vectors (Promega, Madison, WI). Recombinant plasmids were screened by colony hybridization. DNA from recombinant plasmids was purified using Qiagen chromatography and verified by restriction analysis. Sequence of V3 was obtained using Sequenase version 2.0 (U.S. Biochemicals) and the ABI 373A system.

Sequence Analysis

Nucleotide sequences were merged and translated in Gene Runner 3.0 for Windows (Hastings Software, 1994) and aligned using COMPARE in the DNA sequence alignment editor 2.4 (Dr. Alan Goldin, Department of Biology, Cal Tech, 1994). Phylogenetic analysis was performed using neighbor-joining and Kimira two-parameter distance matrix programs in the PHYLIP package (J. Felsenstein, 1993). Bootstrap analysis, based on 100 bootstrap trees, was performed to determine the confidence levels of branching and branch values were superimposed on the neighbor-joining trees. Trees were constructed in the program TREEVIEW version 1.40 (Roderic M. Page, 1997) and were further refined in Harvard Graphics version 1.03 (Software Publishing, 1992).

Genebank sequences were submitted to Genbank; accession numbers are pending.

RESULTS

Clinical Characteristics of Patients (Table I)

The two mothers were 32 and 25 years of age, respectively, at the time their initial viral isolates were obtained. Mother MM had advanced disease and died soon after her sole virus isolate was obtained. Mother NC was asymptomatic but moderately immunosuppressed. All the children were younger than 3 years of age at the start of the study and ranged in age from 31–53 months at the time of collection of the last specimen. In each of the two families, one child was symptomatic (CDC class B1 or C3), and the other had a milder form of HIV infection (CDC class A1 or A3), consisting of nonspecific signs and symptoms. Patient KC developed her first AIDS-defining illness (LIP) at the age of 18 months. Soon thereafter, she developed *P. carinii* pneumonia and survived. She and the other three children showed little further clinical progression. KC and LC are still alive. DM and RM were doing well when last seen at our clinic in July 1995 at ages 49 months and 40 months, respectively. During the study, all patients were treated with various reverse transcriptase inhibitors, alone or in combination, as they became available for clinical use. None received protease inhibitors.

Viral Replication in MDM and PBMC

In PBMC, viruses from family 2 replicated to titers that were 10- to 1,000-fold higher than those from family 1 but all reached maximal titers by day 8 of culture (data not shown). Peak antigen release ranged over 3 logs in dilutions of PBMC culture supernatants, from as low as 10^{-2} in cultures from patient DM to as high as 10^{-5} in cultures from patients NC and KC. Isolates that were related by family displayed no greater than a 10-fold difference in extracellular virus production. Levels of viral replication were reproducible in PBMC from three independent seronegative donors.

Patterns of growth of isolates in MDM were unrelated to their growth in PBMC (Fig. 1). For example, viruses from family 2 that grew to high titer in PBMC showed "slow/low" growth patterns in MDM. The converse was true for viruses from family 1, which replicated rapidly and to moderate levels in MDM, but less well in PBMC (compared with those from family 2).

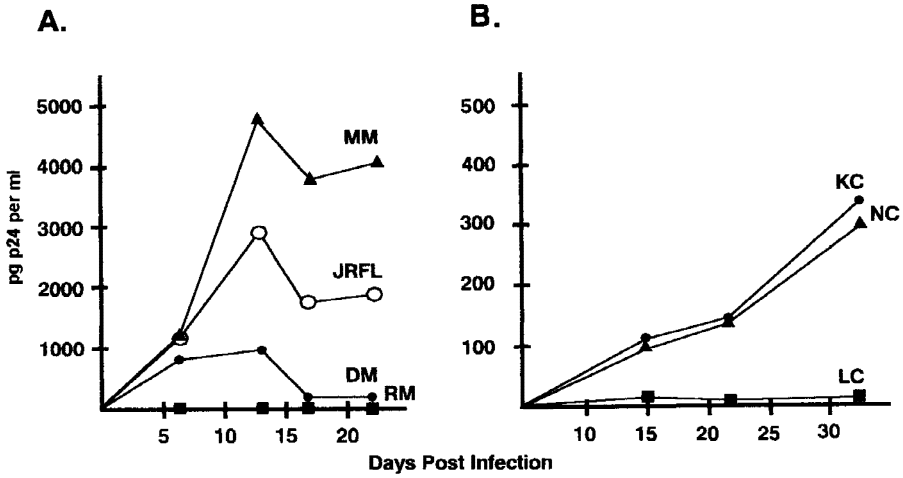

Fig. 1. Replication of virus isolates from patients in each of two families in cultures of monocyte-derived macrophages (MDM). 5×10^6 MDM were infected with $10^{2.0}$–$10^{3.0}$ ID_{50} of the first of six patient isolates of human immunodeficiency virus type 1 (HIV-1). Release of extracellular virus was measured up to 32 days after infection by monitoring p24 antigen levels in culture supernatant fluids. Isolates from families 1 and 2 are displayed in **A** and **B,** respectively. The JR-FL strain of HIV-1 is macrophage-tropic and was used as a positive control. The scales on the y-axes of **A** and **B** are different.

Sequential samples from individual patients behaved similarly in culture. Replication characteristics of all viruses were similar in MDM derived from three independent donors, ruling out donor bias.

Among those viruses from four of six patients that replicated in macrophages, two characteristics, namely peak replication and replication kinetics, segregated by epidemiological linkages. Viruses from MM and DM produced peak p24 levels ranging between 1,000 and 5,000 pg/ml, after 12 or 13 days of MDM culture (Fig. 1A). In particular, MM virus replicated to levels in MDM that were 2- to 5-fold higher than those of the positive control virus JR-FL. In contrast to viruses from family 1, viruses from KC and NC in family 2 replicated more slowly and only reached levels of 325–350 pg/ml of p24 after more than 4 weeks in MDM culture (Fig. 1B). As in PBMC, sequential samples behaved similarly. Viruses from children RM and LC failed to grow in MDM (Fig. 1A,B).

Syncytium Induction and Viral Replication in Continuous Cell Lines

The virus strain recovered from mother MM was unique among the 15 virus isolates in its ability to induce syncytium formation (SI) in MT-2 cells (Table II). MM virus was also the only isolate among the 15 that replicated in MT-2 cells. In addition, virus from MM and from three additional patients (DM, KC, LC) produced syncytia in PBMC cultures. By contrast, virus isolates from asymptomatic children RM and NC failed to replicate or to induce syncytia in MT-2 cells (NSI) or in PBMC. NSI viruses were isolated over periods of months from children with varying degrees of HIV-associated disease (CDC classes A, B, and C) and immunocompromise (CDC classes 1 and 3).

In an effort to assess virus replication in an expanded panel of target cells of diverse origin, two additional T-cell lines (H9 and SupT1), as well as U937 cells and X50-7.8 cells, were evaluated. MM virus failed to replicate in H9 cells, but did replicate to different levels in SupT1, X50-7.8, and U937 cell lines (Table II). Peak amounts of MM progeny virus were produced by days 8–11 postinfection in all three cell lines, although the titer of virus released from X50-7.8 cell cultures exceeded by 1 or 2 $\log_{10}$ the titers produced in U937 or SupT1 cell lines, respectively (data not shown). Extended culture (i.e., >11 days) of isolates in X50-7.8 or U937 cells showed no further evidence of replication by any of the viruses. By contrast, extended cultivation of SupTI co-cultures resulted in production of low levels of p24 by the second of three isolates from the child DM and all three virus isolates from child KC. In contrast with the extended host-cell range displayed by the MM virus isolate, none of 14 isolates from the remaining five patients replicated extensively in any of the continuous cell lines (Table II).

Co-Receptor Usage

Isolates from four patients (MM, DM, NC, KC) released moderate to large amounts of viral antigen into culture fluids of transfected HOS-p.BASE-puro cells (Table II). Isolates from patients DM, KC, and NC (all macrophage-tropic) replicated only in HOS-CD4.CCR5 cells. In cultures of KC virus, p24 antigen was detected in culture supernatants on days 7 and 10; in cultures of NC virus, p24 was detected only on day 10. The isolate from mother MM replicated to high titer in HOS cells bearing the receptor for CD4 alone as well as in HOS cells co-transfected with CD4 and each of four co-receptors: CXCR4, CCR5, CCR3, and CCR2b; p24 antigen was detected in supernatants of all five transfected HOS cell lines beginning on day 3. The isolates from RM and LC, both primary lymphotropic, replicated in Jurkat cells transfected with CCR5, but not in Jurkat cells expressing CXCR4 alone (K. Peden, personal communication). Also, the RM and LC isolates replicated minimally, or not at all, in HOS.CD4.pBABE-puro-CCR5 cells. Except for the isolate from mother MM, no isolate from the other five patients replicated in HOS cells transfected with CD4 alone. Finally, none of the six isolates grew in HOS cells not transfected with the gene for CD4 (data not shown).

TABLE II. Classification and Biological Characteristics of Representative Primary HIV-1 Isolates from Two Mothers and Their Children

Isolates	Predominant phenotype	Viral replication: Primary cells		Viral replication: Cell lines[a]					Syncytium formation	Syncytium formation: Cell lines[a]					Co-receptor use: HOS.CD4.pBABE.puro	With receptor			
		PBMC	MDM	X50-7.8	H9	SupT1	U937	MT-2	PBMC	X50-7.8	H9	SupT1	U937	MT-2	Alone	X4	R5	R3	R2B
Family 1																			
MM (mother)	Dual tropic	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+
DM (child)	Macrophage tropic	+	+	−	−	+/−[b]	−	−	+	−	−	−	−	−	−	−	+	−	−
RM (child)	Primary lymphotropic	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	(±)[c]	−	−
Family 2																			
NC (mother)	Macrophage tropic	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
KC (child)	Macrophage tropic	+	+	−	−	+/−[b]	−	−	+	−	−	−	−	−	−	−	−	−	−
LC (child)	Primary lymphotropic	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	(±)[c]	−	−

PBMC, peripheral blood mononuclear cells; MDM, monocyte-derived macrophages; X4, CXCR4; R5, CCR5; R3, CCR3; R2B, CCR2B.
[a]Replication and syncytium formation were measured in five cell lines, after brief and extended (>11 days) culture.
[b]Three isolates from KC, and 1 of 3 from DM, replicated only in SupT1 cells after 11 days of culture.
[c]Isolates from patients RM and LC grew minimally or not at all in HOS.CD4.pBABE.puro CCR5 cells. However, isolates from both patients replicated in Jurkat cells transfected with CXCR4 and CCR5, but not in Jurkat cells transfected with CXCR4 alone.

Phylogenetic Analysis of *env* V3 Sequences

To establish the genetic linkages among viruses from different individuals in each family and to compare *env* sequences between viruses retrieved in culture to those derived from uncultured patient PBMC, phylogenetic trees were constructed from V3 nucleic acid sequences. Viruses from different individuals clustered to reflect the epidemiological (family) linkages among viruses (Fig. 2). Viruses from each person were more closely related to family members than to viruses from unrelated individuals. Viruses recovered after culture of infected cells clustered with viruses found in uncultured PBMC from each patient, indicating that culture of isolates in PBMC did not select for the outgrowth of rare V3 variants (Fig. 2).

V3 Characteristics and Phenotype

The mean net positive charge among 44 V3 loops in uncultured PBMC from all patients but MM was 3 (range 2–5) (Table III). Basic amino acid substitutions were never detected at positions 11, 13, 25, or 32 in the V3 loops. By the dual criteria of low net positive charge and the position of basic amino acids in the V3 sequences, viruses from each of the five individuals would be classified as NSI, which corresponded with NSI phenotype in MT-2 cells. There was also concordance between the characteristics of the V3 amino acids and a predominant M-tropic phenotype in MDM culture for viruses from DM, NC and KC. However, V3 charge and NSI phenotype did not invariably correspond to M-tropism. For example, the V3 amino acid profiles did not accurately describe the phenotype of the LC or RM virus isolates, which grew efficiently in PBMC, but which failed to replicate in MDM or T-cell lines, or in HOS cells expressing CXCR4 or CCR5.

Dual-Tropic Viruses from Mother MM

The host cell range of the MM isolate included MDM cultures, as well as a variety of transformed cells of lymphocyte and monocyte lineages. In addition, the MM isolate replicated in cells expressing any one of a spectrum of co-receptors. These features might have reflected a mixture of viruses or, alternatively, a virus with dual-tropic characteristics. To distinguish between these two possibilities, the V3 loops of multiple clones of PCR products were sequenced. MM virus sequences contained V3 loops with a net positive charge of 5, reflecting a basic amino acid substitution at position 24 and replacement by threonine for the acidic amino acid found at position 25 in reference virus JR-FL or in the viruses from MM's children (Table III). The V3 charge characteristics of MM viruses were unperturbed by the type of cells in which the viruses were cultured; that is, MM viruses recovered in PBMC culture displayed essentially homogeneous V3 loops that were indistinguishable by charge analysis from viruses replicating in MDM cultures or in continuous cell lines of T-cell, B-cell, or monocytic origin. Therefore, the isolate from patient MM was dual-tropic, demonstrating

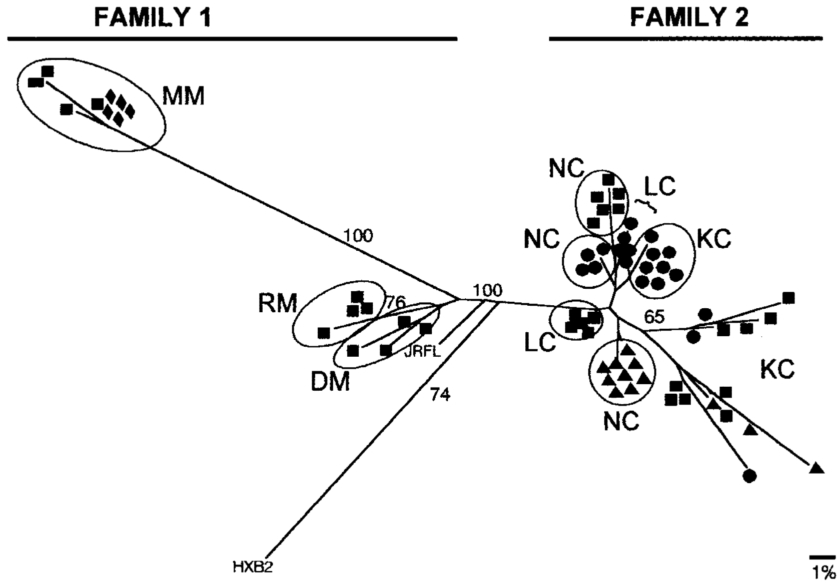

Fig. 2. Phylogenetic analysis of HIV-1 V3 sequences. Family 1 includes MM, DM, and RM; family 2 includes NC, KC, and LC. Numbers on branches represent bootstrap values from 100 replicate trees. ●, patient PBMC; ■, cultured PBMC; ▲, cultured MDM; ▬ Sup T1 cells; ♦, U937. For family 1, the mean intrafamilial distances among the V3 sequences were 3.9 ± 1.7% to 12.4 ± 3.4%; for family 2, the mean intrafamilial distances were 2.0 ± 0.2% to 4.2 ± 0.8%.

the ability to infect both MDM and a variety of mononuclear cell lines (and to utilize four different co-receptors), rather than harboring mixtures of viruses with either a macrophage- or T-cell line-tropic phenotype.

DISCUSSION

Data from this pilot study suggest strongly that differential growth of viruses in MDM is a particularly useful phenotypic feature that can be used to discriminate among viruses with otherwise similar NSI phenotypes. The current study and results from experiments in progress, show that HIV isolates from pediatric patients with advancing disease show a greater tendency to grow in MDM than isolates from less symptomatic patients [Anders et al., 1998]. There was no variation in this observation among isolates collected sequentially from individual patients whose clinical states remained essentially unchanged. Of particular note was our observation that none of the six isolates from the two asymptomatic children grew in MDM, whereas nine isolates from four symptomatic patients did grow in MDM. These data contradict the widely held belief, albeit generated principally from studies in adults, that M-troic strains of HIV are uniformly associated with primary or early-stage infection. Our data also suggest that progression of HIV disease in mothers and children is accompanied by evolution in virus phenotype from primary lymphotropic to macrophage-tropic to dual-tropic. Dual-tropic virus may not always be an evolutionary intermediate between macrophage-tropic and T-cell tropic virus, as has been suggested, but rather a final incarnation that follows in the wake of both M-tropism and T-cell tropism [Doms and Peipert, 1997]. These results are consistent with a model in which viral phenotype develops from a progressive accumulation of characteristics, rather than from discrete “switches” in virus populations, and the ultimate selective outgrowth of some isolates as compared with others [Sakai et al., 1988].

Our data also provide evidence that genetically and epidemiologically related NSI isolates of HIV-1 derived from infected children and their mothers are biologically heterogeneous. Several small cross-sectional studies of pediatric HIV isolates have previously demonstrated that NSI viruses are associated with a spectrum of disease [Spencer et al., 1994; Fitzgibbon et al., 1998]. However, these studies did not assess extensively other phenotypic and genetic features of NSI isolates that might account for their association with varying clinical states. One rationale for conducting the current study was to examine the degree of heterogeneity among genetically related NSI viruses, particularly those collected prospectively from patients with different stages of disease. We succeeded in uncovering phenotypic variation by infecting with a standard viral inoculum an expanded panel of cell types in a wider variety of assays than is conventional. All analyses were done directly; we did not make any a priori assumptions about biologic features that might be linked, e.g., M-tropism and NSI phenotype or syncytium formation and actual replication of isolates in MT-2 cells. Along with our observation that NSI viruses are not uniformly M-tropic, we also found that the M-tropic phenotype is not uniformly associated with a particular V3 loop amino acid profile (charge and sequence).

For the dual-tropic and macrophage-tropic viruses, our analyses demonstrated predictable associations between the predominant phenotype displayed in vitro and replication within cells bearing the co-receptors CXCR4, CCR5, CCR3, and CCR2b. The dual-tropic virus used all four co-receptors and the macrophage-tropic strains used CCR5. Because HOS cells naturally express small amounts of the CXCR4 co-receptor, we cannot be certain whether the MM isolate used CXCR4 exclusively to enter all transfected HOS cells or wheth-

TABLE III. V3 Genotype Characteristics of Viruses

Virus	Phenotype[a]	Cell Type[b]	No. of Clones[c]	V3 Amino Acids[d]	Net + Charge[e]
Control Viruses				NCTRPNNNTRKSI HI GPGRAFYTTGEIIGDIRQAHC	
JRFL	MDM			-▨-◆-----◆◆-- -- ---◆-------□---□-◆---■	3
89.6	DUAL			-▨-◆-----◆◆◆- -- ---◆-----◆◆-----□-◆---■	7
HXB2	TCL			-▨-◆-----◆◆◆-◆--◆---◆-------◆ -----◆---■	9
Family 1					
LC	LYMPH	PBMC	1	-▨-◆------◆-- -- ---G-------A---□-◆---■	2
			3	-▨-◆-----◆◆-- -- ---G-------A---□-◆---■	3
		C-PBMC	8	-▨-◆-----◆◆-- -- ---◆-------A---□-◆---■	4
NC	MDM	PBMC	9	-▨-◆-----◆◆-- -- ---G-------A---□-◆---■	3
		C-PBMC	5	-▨-◆-----◆◆-- -- ---A-------A---□-◆---■	3
			2	-▨-◆-----◆◆-- -- ---◆-------A---□-◆---■	4
		C-MDM	9	-▨-◆-----◆◆-- -- ---◆-------A---□-◆---■	4
KC	MDM	PBMC	1	-■I◆-----◆◆-- -- ---G-------A---□------■	2
			1	-▨-◆-----◆◆-- -- ---G-------A----------■	3
			5	-▨-◆-----◆◆-- -- ---G-------A---□-◆---■	3
			2	-▨-◆-----◆◆-- -- ---◆-------A---□-◆---■	4
		C-PBMC	6	-■I◆-----◆◆-- -- ---◆-------A---□-◆---■	4
			3	-■I◆-----◆◆-- -- ---◆-------A------◆---■	5
		C-MDM	3	-■I◆-----◆◆-- -- --□◆-------A---□-◆---■	3
			6	-■I◆-----◆◆-- -- ---◆-------A---□-◆---■	4
Family 2					
RM	LYMPH	C-PBMC	10	-▨-◆-----◆◆-- -- ---◆-------□---□-◆---■	3
DM	MDM	C-PBMC	10	S■I◆-----◆◆-- -- ---◆-------□---□-◆---■	3
MM	DUAL	ALL	10	-▨-◆-□--I◆◆◆- -- ---◆------◆- -□-◆---■	5

[a]MDM, replicates in primary lymphocytes and monocyte-derived macrophages; LYMPH, replicates exclusively in primary lymphocytes; DUAL, replicates in primary lymphocytes, MDM, and cell lines; TCL, replicates in primary lymphocytes and T-cell lines.
[b]V3 genotype was determined for viruses in different cell types. PBMC, peripheral blood lymphocytes from patients; C-PBMC, cultured PBMC; MDM, cultured MDM; ALL, primary cells and cell lines.
[c]Number of clones with V3 sequences that were similar by amino acid charge analysis.
[d]Amino acids with positive charge (K or R) are designated by a diamond; negative charge (D or E) with a square. Pentapeptide at the tip of the V3 loop is indicated by a bar.
[e]Net positive (+) charge is calculated by formula (K + R) − (D + E).
Note: The amino acid sequences shown in grey shading indicate N-linked glycosylation motifs.

er each of the other co-receptors contributed to the process of attachment, fusion, and entry. By contrast, the primary lymphotropic strains used the CCR5 co-receptor sparingly or not at all, depending on the transfected cell type (HOS vs Jurkat) employed in the assay. These primary lymphotropic strains also may have used CCR5 to gain entry into PBMC, or they may have used receptors/co-receptors or cellular binding proteins (e.g., LFA-1, ICAM-1, mannose-binding protein) other than those assessed in the current study [Ezekowitz et al., 1989; Hansen et al., 1991; Rizzuto and Sodroski, 1997].

On the basis of clinical stage, we anticipated that mother MM would carry SI virus. By contrast, based on the relatively young ages of the four children, we thought it equally unlikely that any of the children would carry SI strains. The results were as expected. The only SI isolate we retrieved was from mother MM; the other 14 isolates, albeit from children at different clinical stages, were all NSI. Using the traditional system of nomenclature, NSI viruses were categorized as such by their inability to replicate in MT-2 cells. However, some predicted NSI viruses did form syncytia in PBMC. Phenotyping viruses based exclusively on their ability to form syncytia in MT-2 cells underrepresents the complexity of syncytium-forming activity that may

exist among viral strains in the HIV-infected patient. The capacity of viruses to form syncytia in PBMC may represent a transitional stage to one of increasing pathogenic potential.

In the current study, detailed phenotypic analysis was accomplished without biological cloning. Therefore, we wondered whether the isolates we obtained from patients might represent mixtures of phenotypic variants that were present in the infected host and whether growth of particular isolates in MDM or cell lines might reflect the proclivity of one variant in such mixtures of viruses to favor a particular cell type. Genotypic analysis helped to resolve that question. For example, the V3 sequences in MDM were very closely related to the V3 sequences in PBMC infected by the same isolates, indicating that viral variants were not selected from virus stocks based on their capacity to replicate in MDM. Furthermore, the V3 genotype of MM virus retrieved from MDM was indistinguishable from the V3 sequence of MM virus in PBMC or cell lines, indicating that MM virus was inherently dual-tropic, rather than a mixture of T-cell tropic and M-tropic viruses. These dual-tropic viruses are distinct biotypes. Their presence in patients may be a marker of even greater pathogenic potential than that of M-tropic viruses. These findings also imply that particular variants of the virus, with their own idiosyncratic biologic behavior and particular genotype, predominate not only at a particular point in time, when they find ready and stable expression in the course of in vitro culture, but also over extended periods of time. There was virtually no variation in phenotype in any of the 15 isolates that we examined over a 2- to 3-year time span.

The similarity of the charge on the V3 loops of 14 NSI isolates suggested to us that all the viruses might also be M-tropic. This was not the case; that is, we did not discover a uniform relationship between M-tropism and V3 loop charge or sequence. Isolates from patients LC and RM had V3 loops with low net positive charge and an amino acid sequence consistent with M-tropism. Nevertheless, they failed to replicate in MDM, in T-cell lines, or in cells carrying the receptors CD4 and each of four co-receptors on their surfaces. We plan future investigations of isolates such as these, which behave unexpectedly, that will include sequence and charge analysis of the V1 and V2 loops, in order to discover if any consistent features of the V1, V2 and V3 loops acting in concert, or of V1 and V2 acting individually, predict phenotype [Groenink et al., 1993].

In conclusion, detailed analysis of a small set of HIV isolates collected prospectively from mothers and their children indicate that, when used systematically in a multifaceted analysis, assessing viruses for replication in MDM is a useful way of discriminating among those that are NSI. We noted an association between advanced disease stage and increasing capacity of NSI HIV isolates to replicate in MDM; this was unanticipated. The pathogenic MDM-tropic phenotype defines a characteristic of NSI viruses that is independent of other biologic and genetic features that we examined, but other likely genetic correlates, perhaps in V2, need to be explored. A large-scale study designed to confirm and extend these findings is in progress.

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